International Journal of Agriculture Innovations and Research
Volume 4, Issue 1, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy) :
Received : 04/05/2015 | Accepted on : 09/05/2015 | Published : 20/08/2015

Classification of Slovenian Wine According to Phenolic Antioxidants and Total Antioxidant Potential

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Abstract – **Nowadays consumers and the food trade are increasingly interested in the geographical origin and the quality of food products. Authentication of geographical origins of wine has become a more important task than ever. The best determination of the region of origin is possible on the basis of the analytical profile of wine. An attempt was made to find useful chemical markers, based on the analysis of individual phenolic antioxidants and total antioxidant potential of wine.**

Principal component analysis (PCA) and multivariate canonical discriminant analysis (DA) were used to classify 196 Slovenian wine samples (112 red, 64 white and 20 rosé wines and mixtures of red and white wines) according to type of wine and viny region based on content of gallic acid, quercetin, (+)-catechin, (-)-epicatechin, vanillic acid, *trans*- and *cis*-resveratrol, 3,4-dihydrobenzoic acid, sinapic acid, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, ellagic acid, caftaric acid and total antioxidant potential (TAP). Determination of phenolic antioxidants was accomplished by HPLC with mass spectrometric and UV/VIS detection. TAP was obtained with the spectrophotometric method described by Singleton and Rossi (TAP_{SP}) and chemiluminometric method using ABEL test kit with Pholasin (TAP_{CL}).

Discriminant analysis of the variables studied made it possible to establish differences in type of wine and viny region. The most meaningful variables for the classification were TAP_{SP} and content of HPLC-UV/VIS determined vanillic acid, 3,4-dihydrobenzoic acid, *p*-coumaric acid and (+)-catechin.

Keywords – **Classification, Discriminant Analysis, Geographic Origin, Phenolic Antioxidants, Wine.**

I. Introduction

Slovenia lies in an ideal winegrowing climate zone with the Adriatic Sea as a part of its western border and the Alps in northern border. The southern slopes of the Alps and their rolling foothills offer a large number of good viticultural sites that are grouped into three viny regions differing in microclimate, soil composition and viticultural tradition: Podravje, Posavje and Primorje. The Primorska winegrowing region is located in the west part of Slovenia where it bordered with Italy and the Adriatic Sea. It is Slovenia's most internationally known region and, though predominately a white wine producer, the region is responsible for most of Slovenia's red wine production. The Posavje winegrowing region with three districts is located in south of Slovenia, bordered by Croatia. This is the region where the French influence affected the local viticulture more than in any other Slovene winegrowing region; consequently, Posavje is primarily known for its blended wines. The Podravje winegrowing region covers the northeastern part of Slovenia, bordered by Austria and Hungary. It is divided into two districts with their specific characteristics. With such a wide variety of ampelographic conditions, Slovene viticulturists have been able apply and customize vines, winegrowing, and methods of making wine from all parts of Europe.

Wines are known to contain many biologically active compounds. The amounts and compositions of these compounds depend on the type of grapes and their degree of ripeness and the climate and soil of the viticultural area, as well as vinification techniques [1]-[3].

The phenolic compounds usually found in wines are gallic acid, catechin, epicatechin, resveratrol and quercetin. Resveratrol, a prominent representative of polyphenols present in fresh grapes and wines, has a pronounced biological activity. Resveratrol has cardio protective effect, because it reduces the susceptibility of low-density lipoproteins to lipid peroxidation (antioxidant effect) and shows a cancer preventing activity [4], [5].

The assessment of wine authentication and typification is a critical issue that has gained a lot of interest all around the world. The study and characterization of different wine varieties of various origins has great importance. Different kinds of fraud, for example the dilution of wines with water, addition of coloring substances, blending with, or replacement by, wine of a lesser quality of denomination, mislabeling, fraudulent misrepresentation of cultivar and geographic origin were known as adulteration.

Phenolics constitute promising class of compounds widely used to categorize wines. Different chemometric procedures have been applied in order to establish criteria for geographical differentiation of wine [6].

Reference [7] shows the distinction between organic and conventional produced wines on the basis of concentrations of phenolic compounds and spectral data. Reference [8] classified wines according to content of metals, volatile compounds and polyphenols. The obtained results indicated a basis for good differentiation between the wines produced in nearby geographical areas. Reference [9] shows classification of red wines on the basis of sensory and chemical analyses. A better classification was achieved on the basis of a chemical data set (major acids, alcohols, esters, pH, total phenols and color). Classification of Greek wines according to geographical origin emerged anthocyanins as a crucial factor in terms of red wine classification, whereas data of phenolic content did not allow any valid clustering of wines [10]. PCA of polyphenols and biogenic amines in Hungarian wines was studied in [11]. The plots of component loadings showed significant groupings for concentrations of polyphenols. Reference [12] discussed content of several polyphenols in samples of red wines from different denominations of origin in the Canary Islands, Spain; HPLC with UV and fluorescence detection was used.

A good differentiation among wines according to their production area was obtained using linear discriminant analysis. For classification of some Australian wines, as in [13], PCA and DA were used to classify the wines according to region of production. Separation between regions was achieved after HPLC analysis with UV and chemiluminescence detection and key components leading to discrimination of the wines were identified. DA correctly classified 100% of the white wines and, overall 91% of the red wines. Reference [14] applied classical multivariate analysis techniques for the classification of Spanish denomination of origin rose wines according to their geographical origin. Nineteen different variables were measured in these wines. The stepwise DA model selected 10 variables obtaining a global percentage of correct classification of 98.8 % and of global prediction of 97.3 %. Another study [15] used liquid chromatography for the fractionation of particular anthocyanins in glycoside form from methanol extracts of red grape skins and solid phase extracts of red wine. By the combination of nuclear magnetic resonance spectroscopy and LC-MS/MS the identification of 13 anthocyanins were obtained. The chemometric methods used were hierarchical clustering analysis and DA. The results of both methods gave 100 % correct classification of wines regarding the vine variety. Reference [16] shows the differences between wines aged through alternative as well as traditional oak barrel systems, i.e., chips stainless steel tanks and staves stainless steel tanks, at the same time and under the same conditions. These differences grew during the bottling period, so that after a 2-year bottling period wines from the three systems became different enough to tell them apart. DA of the variables studied made it possible to establish these differences. The most meaningful variables were yellow colour component, anthocyanins, vanillic acid, protocatechuic aldehyde and epicatechin.

One of author's previous studies compares three different methods for determination of polyphenols in wine: HPLC-UV/VIS, LC-MS/MS and spectrophotometry [17]. It is safe to assume that LC-MS/MS in the multiple reaction monitoring mode is probably most free of interferences, and it could potentially be used as an appropriate reference method. Matrix effects may lead to different results obtained using other methods. In routine work, it is important to understand that HPLC-UV/VIS determinations may be biased to up to 30 % relative to LC-MS/MS determinations. In further work [18], evaluation of the chemiluminometric method (TAP_{CL}) for determination of polyphenols in wine by comparison with LC-MS/MS was discussed. The results were compared with the conventional Singleton-Rossi spectrophotometric method (TAP_{SP}) for determination of total polyphenols.

The purpose of this paper was to classify Slovenian wine according to phenolic antioxidants (gallic acid, vanillic acid, caffeic acid, (+)-catechin, (-)-epicatechin, ellagic acid, *trans*- and *cis*-resveratrol, quercetin, sinapic acid, chlorogenic acid, *p*-coumaric acid, 3,4-DHBA and ferulic acid) and the total antioxidant potential of wine and to confirm the authenticity of wine based on the geographical origin. Because of large quantity of data, a multivariate statistical technique was applied, so it was possible to determine the relationships between the variables and the potential factors that influence them.

II. Materials and Methods

A. Samples

General wine information (e.g. wine type, grape variety, etc) was obtained from the labels on the bottles. Slovenia has three main viny regions: Primorska, Posavje and Podravje. 196 commercially available wine samples (Table 1) were purchased and directly analyzed.

Table 1: Number of wine samples analyzed from the different areas

Viny region	Number of samples
Primorska	68
Posavje	61
Podravje	67
Total	196

B. Analysis of phenolic antioxidants by HPLC-UV/VIS and LC-MS/MS

Gallic acid, ellagic acid, sinapic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, 3,4-dihydrobenzoic acid and *trans*-resveratrol were purchased from Sigma (St. Louis, USA), (+)-catechin hydrate, (-)-epicatechin, vanillic acid, ferulic acid and quercetin dihydrate were purchased from Fluka (St. Gallen, Switzerland). *Cis*-resveratrol was obtained after trans-resveratrol isomerization at 360 nm for 24 h [19]. All reagents and standards were prepared using MilliQ deionized water (Millipore, Bedford, USA).
With both chromatographic methods, we examined the content of phenolics which are the most abundant in wines: gallic acid, (+)-catechin, (-)-epicatechin, *cis*- and *trans*-resveratrol, quercetin, ellagic acid, sinapic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, 3,4-dihydrobenzoic acid, vanillic and ferulic acid.

Stock solutions of standards were diluted in the mobile phase to obtain working standard solutions. Concentrations of the analytes were calculated from chromatogram peak areas on the basis of calibration curves. In HPLC-UV/VIS, identification of the different compounds was achieved by comparison of both the retention times and the absorption spectra with those obtained for the standards. The method linearity was assessed by means of linear regression of the mass of analyte injected vs. its peak area. The repeatability was expressed as standard deviation of three separate determinations.

HPLC-UV/VIS method. The HPLC system Waters 600E was composed of the isocratic pump W600, the autosampler Waters 717+ and the Waters 996 photodiode array detector. Experimental conditions were the following: mobile phase A: 0.1% ortophosphoric acid; mobile phase B: methanol; mixed in a linear gradient as follows: 0 min: 90% A, 10% B; 15 min: 78% A, 22% B; 25 min: 50% A, 50% B; 34 min: 34% A, 66% B; 35 min: 90% A, 10% B; flow-rate: 1.0 mL/min; detection at 210

nm, 253 nm, 278 nm, 303 nm and 335 nm; injection volume: 50 µL; HPLC column: Synergi Hydro RP 150 x 4.6 mm, 4 µm (Phenomenex, Torrance, California, USA), column temperature: 35 °C. Retention times: gallic acid 3.8 min, 3,4-DHBA 6.7 min, (+)-catechin 12.7 min, vanillic acid 15.1 min, caffeic acid 16.4 min, chlorogenic acid 17.0 min, (-)-epicatechin 19.9 min, *p*-coumaric acid 22.7 min, ferulic acid 25.1 min, sinapic acid 25.9 min, *trans*-resveratrol 29.0 min, ellagic acid 29.9 min, *cis*-resveratrol 30.2 min, quercetin 32.4 min. Optimum wavelengths are 210 nm for gallic acid, 3,4-DHBA, (+)-catechin, vanillic acid, (-)-epicatechin, ellagic acid and *cis*-resveratrol, 254 nm for quercetin and 320 nm for caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, sinapic acid and *trans*-resveratrol. All solvents were HPLC-grade and were degassed before use.

LC-MS/MS method. The HPLC system Perkin Elmer PE200 was composed of binary pump, thermostate and autosampler and was coupled to the mass spectrometer 3200 QTRAP LC-MS/MS System with ESI ionization (Applied Biosystems, MDS Sciex, Foster City, USA). The experimental conditions were: mobile phase A: 50 % acetonitrile, 50 % acetic acid (0.5 %); mobile phase B: 2 % acetic acid; gradient elution: 0 min 30 % A, 70 % B; 10 min 30 % A, 70 % B; 30 min 100 % A, 0 % B; 35 min 100 % A, 0 % B; 40 min 30 % A, 70 % B for reconditioning of the system; flow rate: 0.7 mL/min; injection volume: 10 µL; ionization: ESI negative; dwell time 50 ms; MRM transitions: gallic acid 169/125, 3,4-DHBA 153/109, sinapic acid 223/164, vanillic acid 167/123, caffeic acid 179/135, (+)-catechin and (-)-epicatechin 289/245, quercetin 301/151, chlorogenic acid 353/191, ferulic acid 193/134, *trans*-resveratrol 227/185, ellagic acid 301/145, *p*-coumaric acid 163/119 and caftaric acid 311/179. All solvents were HPLC-grade and were filtered and degassed before their use. The wine samples were diluted ten times with the respective mobile phases described above. Typical standard deviations for determinations of the sum of phenolic compounds determined using LC-MS/MS are 0.012 mmol/L for red wines, 0.024 mmol/L for rosé wines and mixtures of red and white wines and 0.070 mmol/L for white wines.

C. Analysis of TAPSP and TAPCL

Spectrophotometry. Determination of TAP_{SP} was performed according to the Singleton-Rossi procedure [20], [21]. The reagent is a mixture of phosphowolframic acid ($H_3PW_{12}O_{40}$) and phosphomolybdenic acid ($H_3PMo_{12}O_{40}$), the absorbance of which was measured after the reaction at 765 nm using a Cary 1E spectrophotometer (Varian, California, USA). The Folin–Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). It contains sodium tungstate, sodium molybdate, ortophosphoric acid, hydrochloric acid, lithium sulphate, bromine, hydrogen peroxide. Briefly, 25 µL of a red, rosé and mixture of red and white wine sample or 250 µL of a white wine sample, 15 mL of distilled water, 1.25 mL of the diluted (1:2) Folin–Ciocalteu reagent, 3.75 mL of a sodium carbonate solution (20 %) are mixed and distilled water is added to make up the total volume of 25 mL. The solution is agitated and left to stand for 120 min for the reaction to take place. The calibration curve was prepared with gallic acid solutions in concentration from 0 to 1000 mg/L. The results were expressed as millimols of gallic acid equivalent per liter. The results for standards were highly reproducible (calibration curve squared regression coefficient >0.9993). All determinations were performed in triplicate. Typical standard deviation for determinations of TAP_{SP} is 0.10 mmol/L for red wines, 0.09 mmol/L for rosé wines and mixtures of red and white wines and 0.02 mmol/L for white wines.

Chemiluminometry. The Abel®-21 M2 antioxidant test kit (Knight Scientific Limited) was used for chemiluminescence measurements of total antioxidant potential - TAP_{CL}. Superoxide, generated in a tube containing Pholasin® leads to appearance of chemiluminescence, which was measured using a micro plate luminometer model Lucy (Anthos Labtec Instruments, Wals, Austria). The producer-prescribed analytical procedure was used. Reconstitution and assay buffer with pH = 7.2 was used in the test kit. The amount of sample was optimized to obtain not more than 90 % and typically 50 % signal inhibition. This signal was then corrected for sample dilution: 10 µL of sample was used, however, red and rosé wines were first diluted with water (1:10) while white wines were not. The results are calculated as TAP_{CL}, expressed as % signal inhibition. We reduce TAP_{CL} for white wines for factor 10, because of sample dilution of red and rosé wines. Typical measurement uncertainty was 3 % for red wines, 2 % for rosé wines and mixtures of red and white wines and 0.2 % for white wines.

D. Statistical analysis

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS 20.0 for Windows (SPSS Inc., Chicago, USA). Factor and multivariate canonical discriminant analyses were carried out with the evaluated compounds.

The number of variables was 20 (gallic acid, (+)-catechin, (-)-epicatechin, *trans*-resveratrol, *cis*-resveratrol, quercetin, 3,4-DHBA, sinapic acid, vanillic acid, caffeic acid, ferulic acid, ellagic acid, *p*-coumaric acid, caftaric acid, chlorogenic acid, TAP_{CL}, TAP_{SP}, Sum LC-MS/MS, type of wine and viny region). With sum of LC-MS/MS we summarize 14 phenolic antioxidants. All variables were mean averaged prior to the analysis.

The factor analysis has been used to concentrate the information in a reduced number of new variables (named factors) that represent the original variables and collect the major part of total variability. The principal components method has been used as a factor extraction method and posterior to that a varimax rotation was carried out to obtain a better interpretation of the factors.

Data for the TAP_{SP}, TAP_{CL} and content of individually determined phenolic antioxidants were processed by analysis of variance as independent variables. Geographic area (viny region), type of wine and the score factors obtained in the factor analysis have been used as dependent variables.

Discriminant analysis is a multivariate technique, which we use to describe group separation in which linear

functions of several variables (discriminant functions) are used to describe or elucidate the differences between two or more groups, leading to the identification of the relative contribution by all variables prior to groups separation and to predict or allocate observations in which linear or quadratic functions of the variable are used to assign an observation to one of the groups [22]–[24].

The stepwise discriminant analysis using the method of Wilks was applied. The first variable to be selected was the one with the smallest value of Wilks' lambda. Lambda is the ratio of the within-group sum of squares divided by the total sum of squares. A large value of Wilks' lambda indicates that the group means do not appear to be different and a small value that group means do appear to be different. Subsequent variables are chosen by the recalculation of lambda for each of the remaining variables and the variable giving the largest change in Wilks' lambda is selected, provided this change is significant when assessed using the F-test. After each new variable is added to the discriminant function, the variables already included in the function are re-assessed and are dropped from the function if the F-test criterion is no longer satisfied (if they no longer contribute significantly to the discrimination). The stepwise operation continues until there are no further variables giving F-values greater than the F criterion. After variable selection has finished, the coefficient is calculated for each variable, together with the constant. These provide an equation which gives a score for each case. In general, the score for one group is positive and the score for the other is negative. The mean discriminant score represents the average of all cases in the group [25]–[29].

III. Results and Discussion

To reduce the number of variables and to investigate the extent of correlation between the TAP_{CL}, TAP_{SP}, individual phenolic antioxidants and sum of LC-MS/MS determined phenolic antioxidants, PCA was performed.

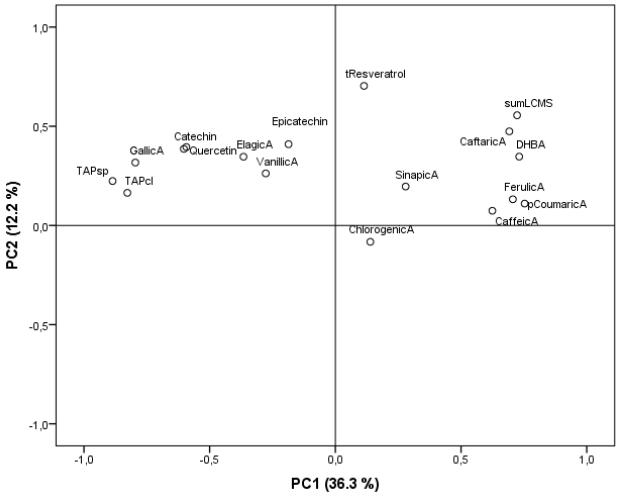

Fig.1. Loading plot for PCA performed with all measured variables: single phenolic antioxidants, TAP_{CL} and TAP_{SP}.

While 36.3 % of the variation is explained by PC1 and another 12.2 % by PC2, comparison of loading factors in Fig.1 shows how different variables might be co-correlated. As it is evident from Fig. 1, CAP_{SP} and CAP_{CL} co-correlate very strong, the impact has also content of HPLC-UV/VIS determined gallic acid. Some smaller is the impact of the content of (+)-catechin, quercetin, ellagic and vanillic acid. There is also a strong correlation between sum of LC-MS/MS determined phenolic antioxidants, caftaric acid, sinapic acid and 3,4-DHBA.

The data was further evaluated by using discriminant analysis. In the first step of DA an F test (Wilks' lambda) was used to test if the discriminant model as a whole is significant. The DA was applied on the raw data consisting of 17 variables (Fig. 2).

Four discriminant functions (DF) were found to discriminate the five types of wines. Wilk´s Lambda test showed that only DF1 and DF2 are statistically significant. DF1 explain 91.4 % of variance and DF2 further 6.8 %. Both discriminant functions are statistically significant; Wilks´ lambdas are small enough, that significance is 0.000. Standardized canonical discriminant function coefficients ware used to compare the relative importance of the independent variables. The importance was assessed relative to the model being analyzed.

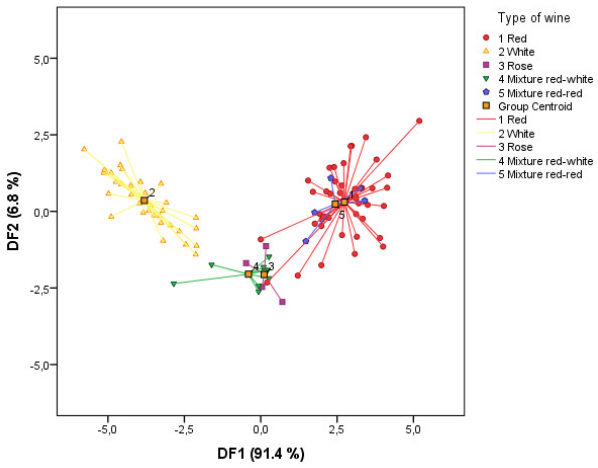

Fig.2. Scatterplot in the space of the first two discriminant functions for total antioxidant potential (TAP_{CL} and TAP_{SP}), individual phenolic antioxidants and sum of LC-MS/MS determined phenolic compounds, considering type of wine.

Maximal first standardized canonical discriminant function coefficient has content of vanillic acid, following by TAP_{SP}, content of 3,4-DHBA and sum of LC-MS/MS, which explains that types of wine differ mostly because of the amount of mentioned phenolic antioxidants and total antioxidant potential. DF2 has maximal standardized discriminant function coefficient by TAP_{SP}.

A table of structure coefficients of each variable with each discriminant function is called canonical structure matrix. Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions are resulting maximal structure coefficients for TAP_{SP} and TAP_{CL} (DF1) and again TAP_{SP} (DF2). The larger the standardized coefficient, the greater is the contribution of the respective variable to the discrimination between groups.

DF1 clearly distinguishes white wines from group of rosés and mixture of red and white wines, which group centroids (projection on abscissa) are really close and further those two groups from group of red wines and mixtures of red wines. DF2 less explicitly distinguishes rosé wines from mixture of red and white wines and group of red, white and mixtures of red wines. 82.3 % original grouped cases of type of wine ware correctly classified (76 % after cross validation).

Discriminant analysis for content of individual phenolic substances, determined with HPLC-UV/VIS and LC-MS/MS methods, TAP_{CL} and TAP_{SP}, considering viny region is represented on Fig. 3.

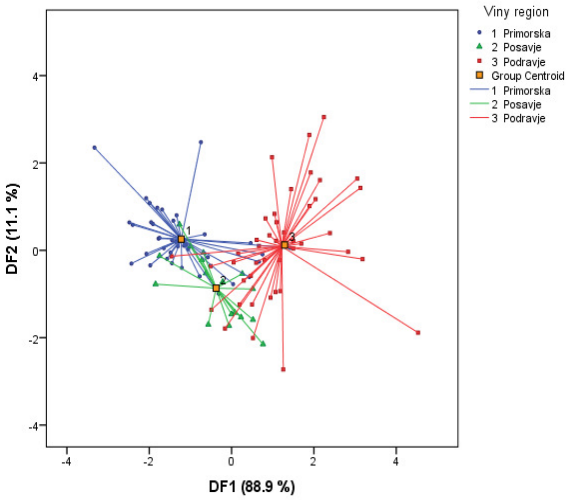

Fig.3. Geographic origin based discriminant analysis of red wine samples, originating from the Slovenian regions Primorska, Posavje and Podravje.

Discriminant analysis shows that DF1 explain 88.8 % of variance, DF2 only 11.2 %. Their canonical correlation coefficients are high. DF1 is statistically significant; Wilks´ lambda is small enough, that significance is 0.000. 72.4 % of original grouped cases of viny regions ware correctly classified (70.4 % after cross-validation). Among the samples of red wines we can most reliably predict wine region Posavje (all originally classified samples of red wines of this wine region were correctly classified), followed by wine-growing region Primorska (92.3 %) and finally wine region Podravje (72.7 %). In Podravje region are actually the most atypical red wines. Maximal first standardized canonical discriminant function coefficient has TAP_{SP}, following by contents of *p*-coumaric acid, vanillic acid and (+)-catechin. Second discriminant function has maximal standardized discriminant function coefficient by content of 3,4-DHBA, following by TAP_{SP}, which explains that viny regions differ mostly because of TAP_{SP} and content of mentioned phenolic antioxidants in wine. Maximal structure coefficients have TAP_{SP}, content of gallic acid, *p*-coumaric acid, TAP_{CL} and caffeic acid. All other determined phenolic antioxidants seem to be unimportant for distinguishing those three viny regions.

IV. Conclusion

Phenolic antioxidant composition and total antioxidant potential of wine in combination with chemometric techniques were used in the classification of Slovenian wine with respect to their type and viny region.

The results of differentiation between single phenolic antioxidants, TAP_{CL} and TAP_{SP} according to type of wine and viny region were 82.3 %, and 72.4 %, respectively.

Types of wine differ mostly because of the amount of vanillic acid, TAP_{SP} and content of 3,4-DHBA. Slovenian viny regions differ mostly because of TAP_{SP} and content of *p*-coumaric acid, vanillic acid, (+)-catechin and 3,4-DHBA. All other determined phenolic antioxidants are unimportant for distinguishing Slovenian viny regions.

Similar to other food industries, the wine industry has a clear need for simple, rapid and cost effective methods for objectively evaluating the quality of wine. The conventional Singleton-Rossi spectrophotometric method provides a rapid tool for wine classification according to geographical origin and could serve as a technique to verify the labeling compliance of the wine.

Acknowledgment

The financial support of the Slovenia Research Agency, Programme no. P1-0153 is gratefully acknowledged.

This study is part of authors PhD work. However, it would not have been possible without the kind support and help of many individuals and organizations. The author would like to extend his sincere thanks to all of them.

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